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Studies on Ca Channels in Intact Cardiac Cells: Voltage-Dependent Effects and Cooperative Interactions of Dihydropyridine Enantiomers

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SUMMARY

We have investigated the effects of two oppositely acting enantiomers of the 1,4-dihydropyridine derivative 202-791 on voltage-dependent Ca channels by combining electrophysiological techniques and binding studies. The (*S*)-enantiomer of 202-791 promoting prolonged openings of single Ca channels, and thereby increasing transmembrane Ba currents, was classified as channel activator. The (*R*)-enantiomer favoring a closed state of the channel, and thereby reducing Ba currents, was classified as a channel blocker. Both compounds shifted the steady state current inactivation curve toward more negative potentials. At holding potentials positive to −20 mV, the Ca channel-activating effect of the (*S*)-enantiomer turned over into a blocking effect. In cells with normal resting potential the combination of the two enantiomers revealed a possible positive cooperative effect resulting in an enhancement of the open state probability of the channels. At depolarized holding potentials the activator enhanced the inhibitory effect of the blocker. Binding studies in intact cells were performed by using the radiolabeled channel-blocking dihydropyridine ^{3}H-(+)-PN 200-110. The results showed a strong increase in binding affinity but no change in binding capacity when the cells were depolarized. Analysis of the interactions of (*S*)- and (*R*)-202-791 with this radioligand indicated stimulation of ^{3}H-(+)-PN 200-110 binding by the (*S*)-enantiomer in polarized cells (membrane potential −38 ± 4 mV). This effect could be attributed to an increase in binding affinity. The (*R*)-enantiomer had no such positive cooperative effect, but acted as a purely competitive ligand. Depolarization to 0 mV increased the apparent affinity of both enantiomers by factors of 38 (blocker) and 12 (activator), but abolished the cooperative effect of (*S*)-202-791 on the binding of the radioligand. Ca ions had little effect on the binding of ^{3}H-(+)-PN 200-110 in polarized cells. However, in the presence of the activating (*S*)-enantiomer, Ca transformed the usual hyperbolic binding isotherm of the radioligand into a strongly sigmoid curve. Sigmoidicity was minimal with 3–5 μM Ca and maximal with 0.5 mM Ca. Together these data demonstrate homotropic and heterotropic cooperative interactions between channel activator and channel blocker. They indicate that at least two high affinity binding sites for dihydropyridines are associated with voltage-dependent Ca channels. Voltage dependence of both—binding affinity and cooperativity—suggests that these binding sites are located close to a structural component of the channel which is involved in the potential-sensitive gating process.

The important role of Ca ions in the regulation of many cellular functions is widely recognized. Ca channels in surface membranes of cells contribute to the regulation of the free cytosolic Ca concentration in various cell types. These channels are integral membrane proteins forming pores through which Ca ions can flow into the cell. The gating of these channels, that is, their opening and closing kinetics, can be investigated by means of the patch clamp technique (1). Application of this method to cardiac cells has provided evidence for two types of Ca channels, T-type and L-type (2, 3). These channel types can be distinguished not only by their respective ion selectivities and by their distinct modes of gating, but also by their different sensitivities to 1,4-dihydropyridines (2, 3). The L-type Ca channel but not the T-type is sensitive to these drugs. Previous studies have shown that the action of dihydropyridines on these channels is strongly voltage dependent (4–6). In the present study we have investigated two enantiomers of the dihydropyridine derivative, Sandoz 202-791, which have opposite effects on cardiac cells (7, 8). One enantiomer [(+)-(*S*)-202-791] acts as a Ca channel activator by prolonging the open state of the channel, whereas the other enantiomer [(−)-(*R*)-202-791] is a Ca channel blocker, i.e., it inhibits channel open-

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ABBREVIATIONS: (+)-PN 200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; (+)-(*S*)- or (−)-(*R*)-202-791, isopropyl 4-(2,1,3-benzoxadiazo-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate; BAY K 8644, methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate.

ings. We have studied the voltage dependencies of these changes in Ca channel gating and compared them with the voltage dependence of radioligand binding of these drugs in tissue-cultured cardiac cells. Our results show that at least two cooperatively interacting binding sites for 1,4-dihydropyridines are present in L-type Ca channels. A preliminary report of these results has been published (9).

Materials and Methods

Radioligand Binding Experiments

Rat heart cell cultures. Hearts from 2–4-day-old rats were used as the source of myocardial cells. To establish primary cultures cardiac ventricles were enzymatically dissociated and the cells were seeded onto glass coverslips according to the methods described by Porzig *et al.* (10). Usually 90–120 coverslip monolayer cultures could be prepared from the pooled ventricles of ~80 hearts. Plating density was kept at about 1×10^6 myocytes/coverslip. Groups of 12 cultures were grown together in one Petri dish in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum. All binding experiments were performed on day 3 of culture. By this time a regularly beating cell layer had formed on each coverslip containing on the average 254,000 ± 9,500 cells.

Receptor binding assay. Binding assays were performed on intact cells at two different membrane potentials. Under control conditions in nominally Ca-free Hank's solution (contents in mM: NaCl, 137; KCl, 5.4; $MgSO_4$, 1.2; Na_2HPO_4, 0.34; KH_2PO_4, 0.44; $NaHCO_3$, 4.2; glucose 5.5; HEPES, 20, pH 7.2 at 37° = NaCl-Hanks'), the mean (±SD) resting potential of cardiac cells within the coverslip cell layer was −38 ± 4 mV ($n = 8$). A membrane potential close to 0 mV was established by replacing most of the NaCl in Hanks' solution by KCl (composition of the depolarizing solution in mM: NaCl, 5.4; KCl, 137; other components as above). Total Ca concentrations in nominally Ca-free solutions measured by atomic absorption spectrophotometry varied between 2 and 5 μM. ^{3}H-(+)-PN 200-110 (Fig. 1) (11) was used throughout to label 1,4-dihydropyridine-binding sites. Equilibrium binding was determined by incubating individual coverslip cultures for 60 or 90 min at 37° in 3 ml of the experimental medium containing 0.01–2 nM ^{3}H-(+)-PN 200-110. The cultures were then washed for 5 sec each in three beakers containing 400 ml of washing solution (10 mM K_2HPO_4, pH 7.5) which was kept at 37°. Control measurements showed that the decrease of the free radioligand concentration in the incubation medium at binding equilibrium ranged between 2 and 12% at 1 or 0.05 nM ^{3}H-(+)-PN 220-110, respectively. After the final wash, the cultures were transferred into wide-neck counting vials (Beckman Poly Q2) and digested with 0.8 ml of a 1:2 (v/v) mixture of absolute ethanol and Solutron (Kontron AG, Zürich, Switzerland). ^{3}H activity was counted in an Intertechnique SL 4000 or a Kontron MR 300 liquid scintillation counter using a Triton/xylol-based commercial scintillator (Kontrogel).

Specific binding was defined as that amount of radiolabel that could be displaced competitively by 1 μM nonlabeled (+)-PN 200-110 or (±)-nisoldipine. Nonspecific binding was not affected by membrane potential changes and amounted to about 17% (depolarized) or 50% (polarized) of total binding at 0.1 nM ^{3}H-(+)-PN 200-110. At 1.5–2 nM, 75% of the label was bound nonspecifically. All experiments were run at least in triplicate.

Because of the light sensitivity of 1,4-dihydropyridine compounds, experiments were generally carried out under sodium light (λ = 440 nM). Glassware was used for all incubation and pipetting purposes to avoid the strong adsorption of dihydropyridines to plastic materials.

Protein determination. From each group of 12 coverslip cultures 2–4 sample cultures were used to estimate the mean protein content per coverslip. The cells were washed free of culture medium, removed from the growth surface by a freeze-thawing cycle, and then homogenized by sonification for 5 sec in a volume of 1 ml of 30 mM NaCl. The protein content of the cell homogenate was measured according to the method of Lowry *et al.* (12) using bovine serum albumin as a standard. In different experiments the protein content per culture varied between 150 and 250 μg.

Data analysis. Maximal binding capacities (B_{max} values), apparent dissociation constants (K_D values), and the concentrations of a nonradioactive competitive ligand required for half-maximal displacement of ^{3}H-(+)-PN 200-110 (I_{50} values) were calculated from computerized nonlinear least squares fits of the data points. The fitting procedure used a BASIC version of the program "MODFIT" published by McIntosh and McIntosh (13) running on a Hewlett-Packard HP 9816 computer. Apparent K_D values were calculated from I_{50} values using the method of Cheng and Prusoff (14). Binding data are presented as means ± standard errors. Student's t test was used to assess the significance of differences between mean values. A probability of $p < 0.05$ was considered statistically significant.

Electrophysiological Experiments

Isolated single ventricular cells from adult rat hearts and primary cultures of cardiac cells prepared from neonatal rat hearts were used for electrophysiological experiments. The isolation procedure of ventricular cells was essentially the same as described elsewhere (15). Briefly, rat hearts were dissected and perfused for 35 min by the Langendorff method with saline solution containing 0.5–1 mg/ml collagenase (Sigma type I or IA) and 0.1–2 mg/ml trypsin (Sigma type IV). The composition of saline was (in mM): NaCl, 137; KCl, 5.4; $CaCl_2$, 2.0; $MgCl_2$, 1.5; HEPES, 10 (pH 7.4); glucose, 20. After perfusion with the enzyme-containing solution, hearts were kept in a storage solution (4°) of the following composition (in mM): KCl, 25; KH_2PO_4, 10; potassium glutamate, 120; glucose, 11; EGTA, 1; ATP, 1; and HEPES, 10 (pH 7.4). Dissociated cells were used within 9 hr after isolation. Primary cultures of myocardial cells were prepared as described above. The cells were seeded at a low density ($1–3 \times 10^5$ cells/ml) on collagen-coated coverslips. Differentiated cells with spindle-like shape and clear striation were used 1–2 days after seeding for Ca channel recordings. Membrane potentials were measured with conventional microelectrodes in monolayers of heart cell cultures prepared exactly as for binding studies.

Single Ca channel currents were recorded by means of the patch clamp method (1). The cells were immersed in solutions containing (in mM) either: potassium aspartate, 125; EGTA, 5; and HEPES, 10 (pH 7.4), or NaCl, 137; KCl, 5.4; $MgCl_2$, 1.5; $CaCl_2$, 2.0; HEPES, 10 (pH 7.4). The temperature of the bathing solution was kept at 28 ± 0.5°. The patch micropipette contained 45 mM $BaCl_2$, 70 mM tetraethylam-

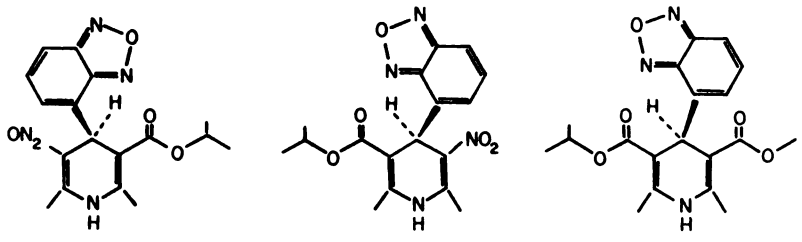

Fig. 1. Chemical structure of the enantiomers of the compound 202-791 and of the radioligand (+)-(S)-PN 200-110. The (+)-(S)-enantiomer of 202-791 is an activator, the two other compounds are blockers of myocardial Ca channels.

monium chloride, and 20 μM tetrodotoxin, buffered to pH 7.4 with 10 mM HEPES. Single channel currents, with Ba^{2+} ions as charge carriers, were measured in the cell-attached patch configuration. Single channel currents and applied voltage pulses were recorded on a tape recorder (Racal 4DS). Current traces were filtered with a two-pole Bessel filter (cut-off frequency 1 kHz at −3 dB). Digitized records (sampling interval 200 μsec) were analyzed by means of a minicomputer (PDP 11/04).

Materials

Newborn rats were obtained from the animal breeding facility of the Institute of Pathophysiology, University of Bern. Tissue culture reagents were obtained from Boehringer Mannheim Corp., Rotkreuz, Gibco, Basel, and Flow Laboratories, Baar, Switzerland. ^{3}H-(+)-PN 200-110 (3.03–3.15 TBq/mmol) was purchased from Amersham UK. Nonlabeled (+)- and (±)-PN 200-110 as well as the two enantiomeric dihydropyridines (+)-(*S*)-202-791 and (−)-(*R*)-202-791 (Fig. 1) were gifts of Sandoz AG, Basel, Switzerland (Dr. R. P. Hof and Dr. U. Rüegg). Other 1,4-dihydropyridine compounds (^{3}H-(±)-nimodipine (5.36–5.56 TBq/mmol), nisoldipine, (±)-Bay K 8644) were gifts of Bayer AG, Leverkusen, FRG (Prof. F.Hofmeister).

Results

Electrophysiological experiments. Ca channels in cardiac cells have been extensively studied over the last few years by means of the patch clamp method. There are at least two types of Ca channels (T-type and L-type) (2, 3). However, only the L-type Ca channel seems to be sensitive to dihydropyridines (2, 3). Fig. 2 shows elementary currents flowing through a single Ca channel recorded from a single cardiac cell from an adult rat heart. The *left column* (*control*) shows 12 consecutive single channel current traces, while the *lowest trace* shows the ensemble average current obtained from the summation of 219 such channel records. The single channel traces show the typical behavior of Ca channel currents when Ba ions are the charge carriers. The channel flickers between a closed (Fig. 2, *solid line*) and an open (Fig. 2, *dotted line*) state. In myocardial cells, Hess *et al.* (6) have defined phenomenologically three different types of Ca channel-gating behavior called "modes." This categorization is also applicable to our observations. Channel openings are often grouped together as bursts (mode 1). Occasionally, the channels do not open at all (see eighth and ninth current traces; mode 0). Very rarely the channel enters a prolonged open state (not shown; mode 2). Addition of the (*S*)-enantiomer of the drug 202-791 facilitates the state of the channel where it frequently stays open for prolonged periods of time (Fig. 2, *right column*). In other words, mode 2 behavior, which is extremely rare in the absence of the drug, is very common in its presence. Due to long channel openings, the ensemble average current is greatly increased in the presence of the (*S*)-enantiomer (Fig. 2, *lowest trace*). A similar effect on open times of Ca channels has been observed with two other dihydropyridines, CGP 28392 and BAY K 8644 (6, 16, 17). By contrast, the (*R*)-enantiomer of the dihydropyridine compound 202-791 shows an effect on single Ca channels which is completely different from that of the (*S*)-enantiomer (Fig. 3). Compared to the controls the channel stays closed (mode 0) for prolonged periods of time. In addition, reopening of the channel is impeded so that openings tend to be clustered at the beginning of the current traces. Correspondingly, the ensemble average current (*lowest traces* in Fig. 3) is not only reduced in amplitude, but the rate of inactivation is also accelerated. It is of interest to note that reopening of the channel is also hindered

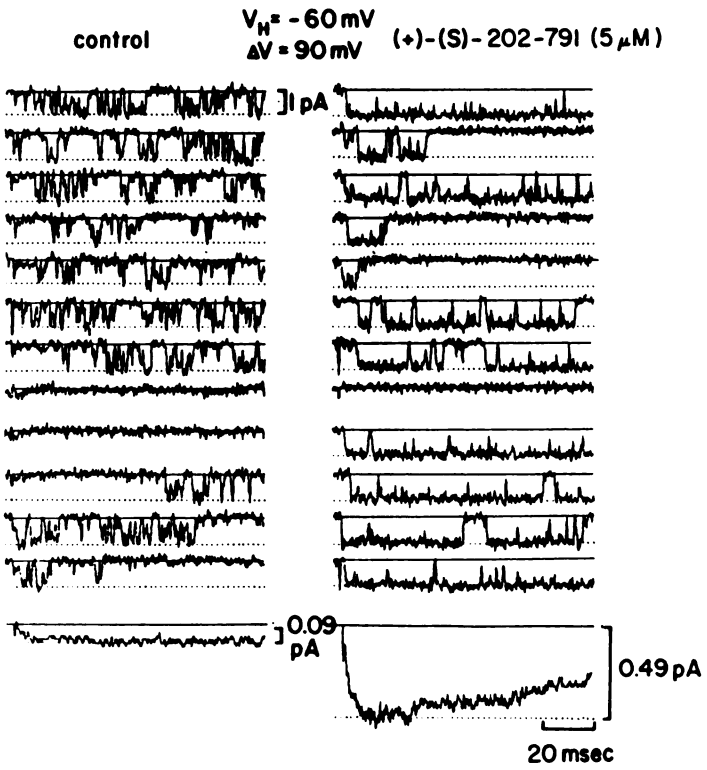

Fig. 2. Elementary Ca channel currents recorded from a cell-attached membrane patch in an adult rat heart ventricular cell. The pipette was filled with 96 mM $BaCl_2$; thus, the current carrier was Ba^{2+}. Depolarizing clamp steps (Δ*V*) of 90 mV amplitude were applied from a holding potential (V_H) of −60 mV. Clamp pulse duration was 100 msec. On the *left side* are shown 12 consecutive single channel current traces obtained under control conditions. The *heavy lines* indicate the closed state of the channel; the *dotted lines* indicate the open state. The *lowest record* is the ensemble averaged current obtained by summation of 219 single channel records as above. The *right side* shows corresponding single channel records and ensemble averaged current in the presence of the channel-activating enantiomer (+)-(*S*)-202-791.

in the presence of activator (*S*)-enantiomer, resulting also in an increased rate of inactivation (Fig. 2, *lowest traces*). These results show that the (*R*)-enantiomer predominantly favors a closed state (mode 0) of the channel, thereby reducing the Ca currents, whereas the (*S*)-enantiomer primarily stabilizes the prolonged open state (mode 2) of the channel which results in an enhancement of the average currents.

Several investigators (4–6) have shown that the effect of certain dihydropyridines strongly depends on membrane potential. We have investigated whether the same holds true with the two enantiomers of the dihydropyridine compound 202-791. Fig. 4 (*upper*) shows the ensemble average Ca channel currents. The membrane potential was always pulsed to +30 mV for 100 msec, whereas the holding potential (V_H) varied between −60 and +20 mV. With increasing depolarization of V_H the current amplitude became smaller, a typical voltage-dependent inactivation behavior (18, 19). Average currents in the presence of the (*S*)-enantiomer were much larger than the control current traces when V_H was held at negative potentials. At −20 mV, the peak amplitude of the currents was about the same, and positive to 0 mV, the currents were virtually abolished. A normalized plot of these data (Fig. 4, *lower*) reveals a marked parallel shift of the inactivation curve by the drug toward more negative membrane potentials. The Ca channel-

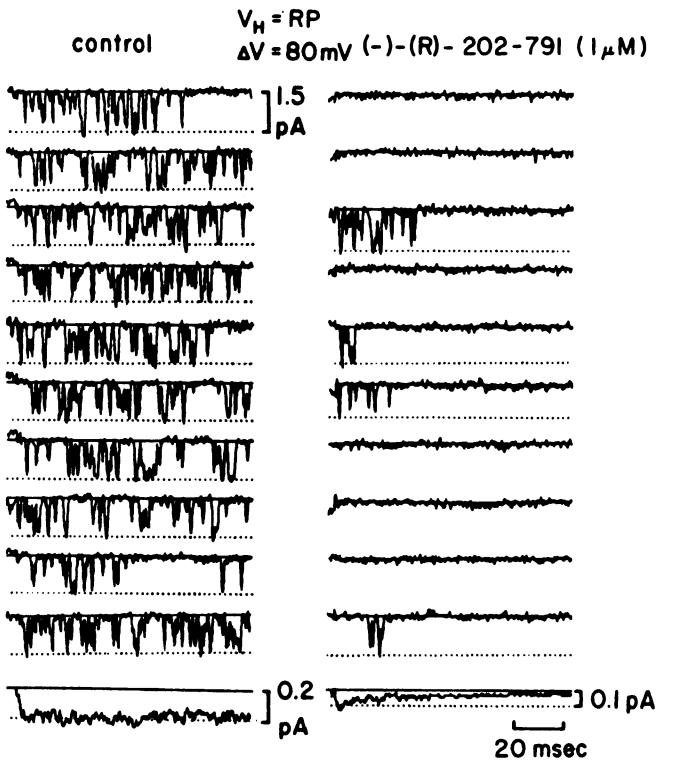

Fig. 3. Elementary Ca channel currents recorded from a cell-attached membrane patch in an adult rat heart ventricular cell in the absence (control; *left*) and presence (*right*) of the channel-blocking enantiomer (−)-(*R*)-202-791. Same protocol as in Fig. 2.

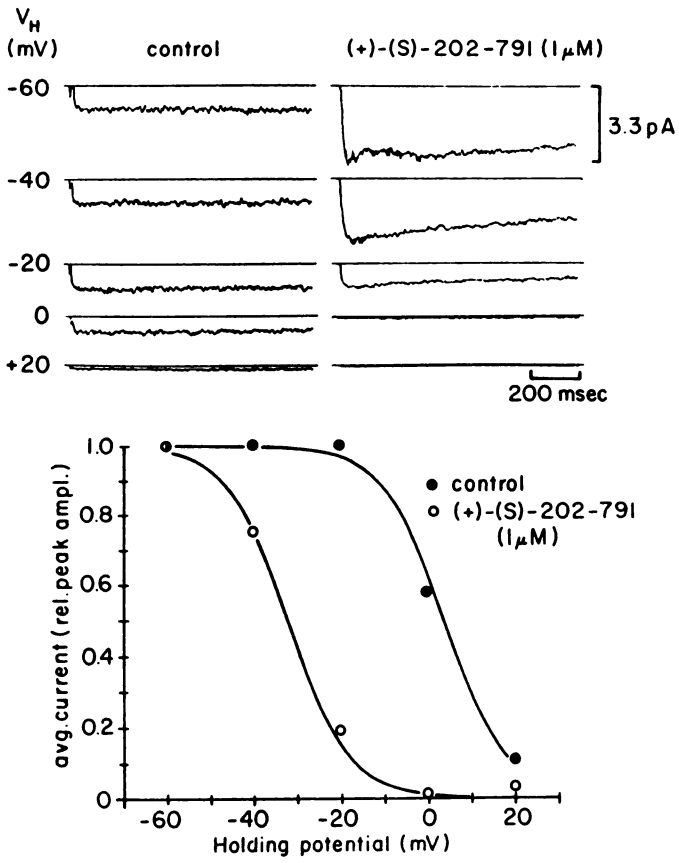

Fig. 4. Effect of (+)-(*S*)-202-791 (channel activator) on the voltage dependence of averaged Ca channel currents measured in adult rat heart cell. Note that the channel-activating effect turns over into a blocking effect at a holding potential of −20 mV (*upper part*). *Solid lines* fitting the normalized data points (*lower part*) were drawn according to: $(1 + \exp[(V_m - V_{0.5})/K])^{-1}$ with $V_{0.5}$ = +4 mV, K = 7 mV (control) and $V_{0.5}$ = −32 mV, K = 7 mV (drug).

blocking (*R*)-enantiomer also causes a shift of the inactivation curve toward more negative potentials (Fig. 5), albeit at much lower concentrations than the Ca channel-activating (*S*)-enantiomer.

Analysis of the corresponding single channel current data with both enantiomers revealed a large increase in the number of traces where no openings occur (mode 0) when V_H is depolarized. Therefore, the current enhancing effect of the (*S*)-enantiomer turns over into a blocking effect at V_H positive to −20 mV (Fig. 4, *upper*), although long openings can still be observed when the channel opens at all. In the case of the (*R*)-enantiomer, mode 0 behavior simply becomes more and more pronounced when the membrane is depolarized. However, at very low concentrations ($<5 \times 10^{-8}$ M) of the (*R*)-enantiomer an increase in the number of traces with long openings can also be observed at negative holding potentials. Results similar to those in Figs. 4 and 5 with the (*S*)- and (*R*)-enantiomers have been obtained in five and six experiments, respectively. The conclusion from these results is that the electrophysiological evidence with the two enantiomers of the compound 202-791 shows an interference of these drugs with Ca channel gating. The (*R*)-enantiomer predominantly favors a closed (mode 0) state of the channel, while the (*S*)-enantiomer primarily stabilizes the prolonged open (mode 2) state.

Since many of the 1,4-dihydropyridines are racemic compounds, it was of interest to combine the (*S*)- and (*R*)-enantiomers of the substance 202-791. Fig. 6 illustrates such an experiment. To our surprise, the greatly enhanced Ca channel activity induced by the (*S*)-enantiomer was not blocked by the (*R*)-enantiomer, but rather increased if the membrane potential was held at its resting level. Flickering of the channels was

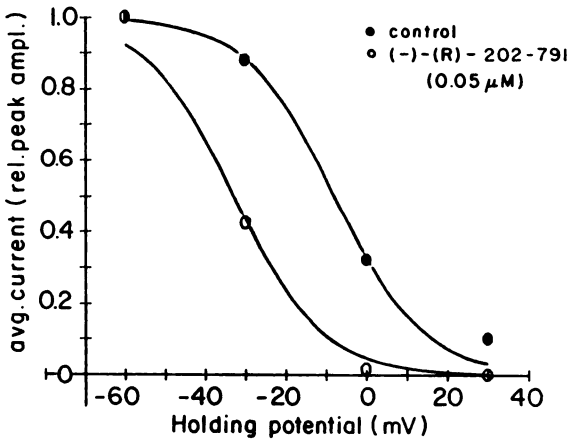

Fig. 5. Effect of (−)-(*R*)-202-791 (blocker) on the voltage dependence of averaged Ca channel currents measured in cultured cardiac cells. Normalized current scale; absolute peak currents (I_{max}) at a holding potential of −60 mV were 2.8 pamp in the control and 1.65 pamp in the presence of the drug. Pulse potential was always +40 mV. *Solid lines* were drawn according to: $(1 + \exp[(V_m - V_{0.5})/K])^{-1}$ with $V_{0.5}$ = −8 mV, K = 12 mV (control) and $V_{0.5}$ = −34 mV, K = 12 mV (drug).

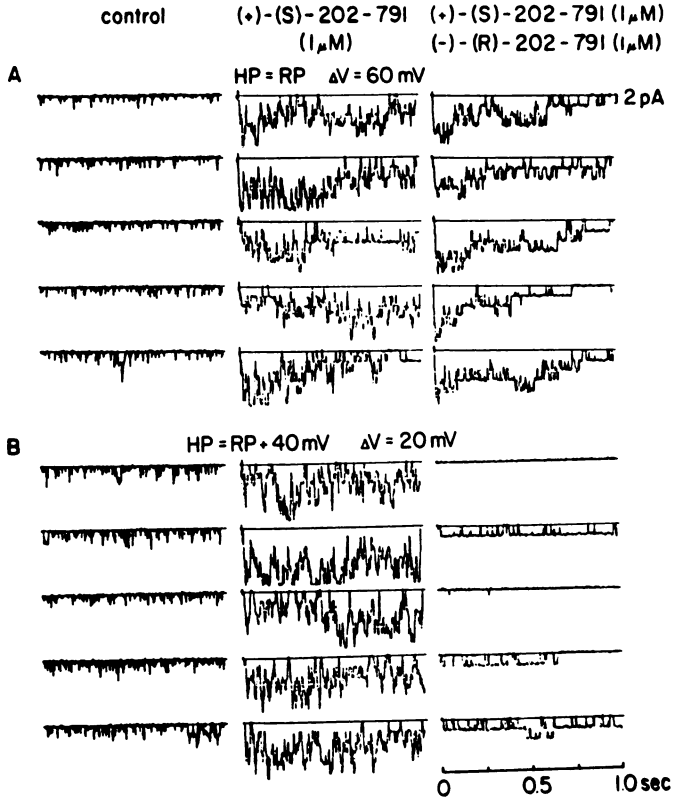

Fig. 6. Single channel current traces recorded from an adult rat heart cell at two different holding potentials in the absence and presence of (+)-(*S*)- and (−)-(*R*)-enantiomers of the drug 202-791. Ba^{2+} ions were the charge carriers through open channels. There were at least six Ca channels in the membrane patch. Single channel currents in the controls are poorly resolved due to the slow time resolution. Increased channel activity in the presence of the drug is due to marked prolongation of the times the channel spends in the open state. A. Holding potential (*HP*) was equal to the resting potential (*RP*), and voltage clamp pulses (ΔV) of 60 mV amplitude lasted 1 sec. B. The holding potential was adjusted 40 mV positive to the resting potential and pulse potentials of 20 mV amplitude were applied. Note the sharp decrease of channel openings at this holding potential when both enantiomers are present in the bathing solution.

reduced and they seem to stay open longer than with the (*S*)-enantiomer alone (Fig. 5, *upper*). If, however, the holding potential was depolarized by 40 mV, a prominent blocking effect occurred due to an increase in the number of failures of the channels to open upon depolarization (Fig. 6, *lower*). This result suggests a possible cooperative interaction between the two enantiomers, thereby promoting either the prolonged open state (mode 2) of the channel when the membrane potential is more negative or a prolonged closed state (mode 0) when the potential is more positive. It also provides strong evidence against the hypothesis of open channel blockade by antagonistic 1,4-dihydropyridines (20, 21).

Fig. 7 shows a more complete analysis of the combined effects of the two enantiomers. In this case we have chosen concentrations of the two enantiomers which were different by a factor of 10, corresponding roughly to the respective half-maximally effective concentrations in cells with normal resting potential. Fig. 7 (*right*) shows average currents obtained from summation of 219 single channel current traces. The mean open times and open state probabilities during the individual traces have been plotted on the *left side* of the figure. The (*S*)-enantiomer alone approximately doubled the size of the ensemble average current. This was due to an increase in the mean open times of the channels and to a corresponding increase in the open state probability. In addition, the number of traces in which the channel failed to open was reduced. If the (*R*)-enantiomer was also added, there was a further doubling of the size of the average current and a further increase in the number of long openings. These results indicate that some cooperativity between the two enantiomers must occur, since a simple additive effect would have reduced rather than increased the average current. This, however, implies a complicated interaction between binding of the enantiomers at sites within the channel that are related to its gating. Further information on this question was obtained by binding studies.

Binding of ^{3}H-(+)-PN 200-110. Equilibrium binding of the Ca channel blocker PN 200-110 is strongly voltage dependent. Fig. 8 compares binding curves obtained under control conditions in Ca-free, NaCl-Hanks' solution (steady membrane potential about −38 mV) and under depolarizing conditions in KCl-Hanks' solution (membrane potential about 0 mV). In both cases the data points could be fitted by assuming a single homogeneous population of binding sites. Depolarization decreased the K_D value for ^{3}H-(+)-PN 200-110 by a factor of 12 from 0.73 ± 0.13 nM to 0.059 ± 0.005 nM, but left the binding capacity unchanged. The B_{max}- values for the specific binding of ^{3}H-(+)-PN 200-110 in controls and in depolarized cells reached 158.9 ± 20.1 and 158.3 ± 6.6 fmol/mg of protein, respectively. These values correspond to about 54,000 binding sites/myocyte.

Since the membrane potential change had been induced by a change in the cation species in the incubation medium, it was essential to test whether the ion composition of the medium per se might affect the binding affinity of ^{3}H-(+)-PN 200-110. In experiments shown in Fig. 9 we have measured specific binding of ^{3}H-(+)-PN 200-110 (0.1 nM) with increasing concentrations of KCl between 2.5 and 137 mM and corresponding decreases of NaCl in the incubation medium. In agreement with the data shown in Fig. 8 the fractional receptor occupancy at 0.1 nM ^{3}H-(+)-PN 200-110 increased gradually from 20 to 105 fmol/mg of protein as the extracellular K concentration ($[K^+]_o$) was raised from 5.4 to 50 mM. This change in $[K^+]_o$ was associated with a drop in membrane potential from about −40 to less than −10 mV. A further stepwise increase in $[K^+]_o$ to 137 nM and a corresponding decrease in $[Na^+]_o$ from 90 to 2.6 mM had little additional effect on membrane potential and also had no further effect on the ^{3}H-(+)-PN 200-110 binding affinity. Therefore, we conclude that NaCl replacement by itself does not account for the observed change in the K_D values.

The depolarization-induced increase in binding affinity was reversible upon repolarization. When K-depolarized cells were first equilibrated with 0.6 nM ^{3}H-(+)-PN 200-110 and then transferred into a large volume of either NaCl- or KCl-Hanks' medium without radiolabel, the ^{3}H-(+)-PN 200-110 dissociation rate was significantly faster in the NaCl-medium than in the KCl-medium. It has recently been shown that membrane repolarization in cardiac sarcolemmal vesicles also causes an increase in the dissociation rate of the dihydropyridine compound ^{3}H-nitrendipine (22).

These results confirm that ^{3}H-(+)-PN 200-110 binding affinity is greatly dependent on membrane potential. They agree with our and other (8, 9) electrophysiological evidence that Ca channels in the inactivated state, when cells are depolarized,

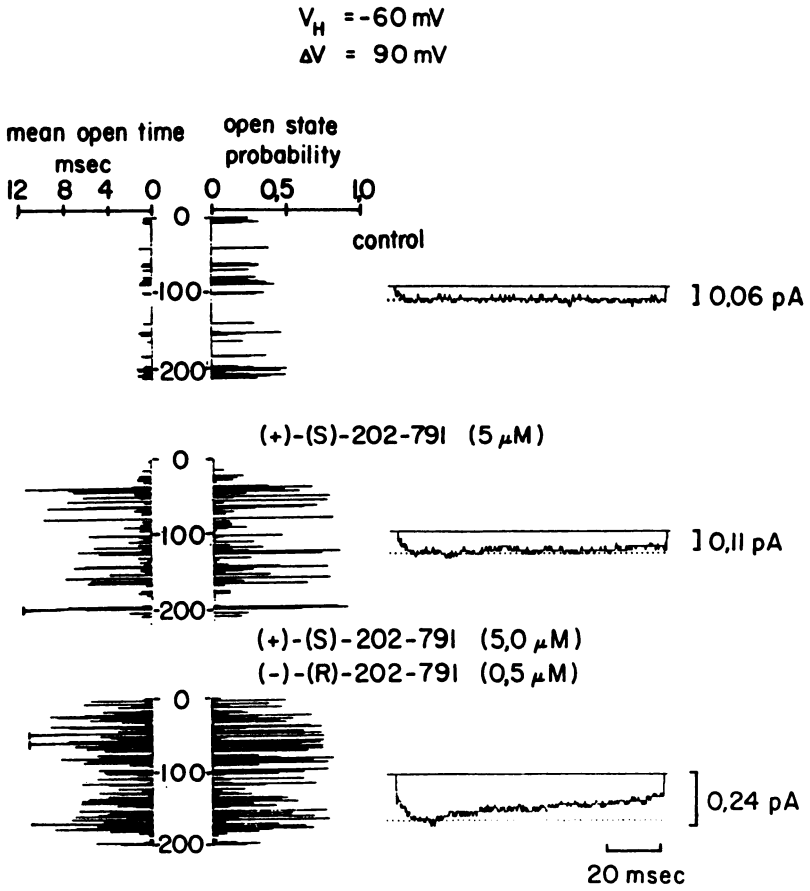

Fig. 7. Ensemble averaged Ba currents (*right*) obtained from summation of 219 single channel current records as illustrated in Figs. 2 and 3. On the *left* are mean open times and the corresponding open state probabilities of each of the 219 single channel current records. The currents were measured in an adult rat heart cell under control conditions (*upper*), in the presence of the (+)-(*S*)-202-791 enantiomer (*middle*), and in the presence of both (+)-(*S*)- and (−)-(*R*)-enantiomers of the compound 202-791 (*lower*).

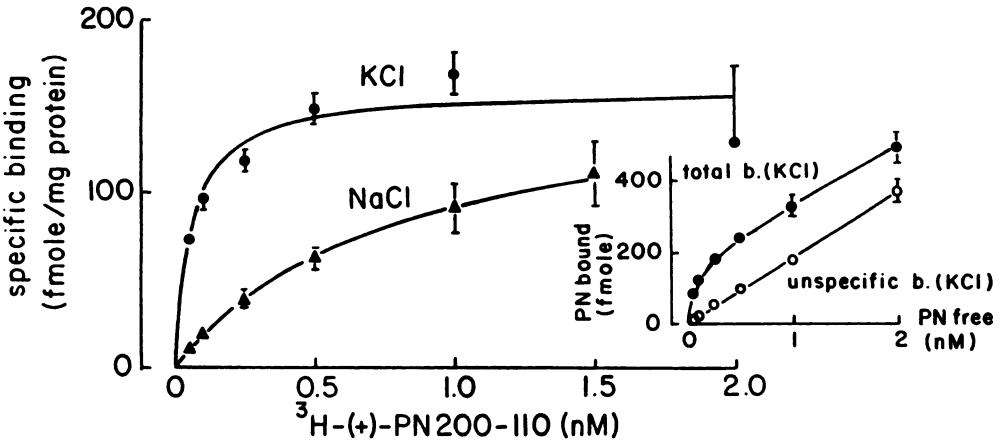

Fig. 8. Effect of K-depolarization on specific binding of ^{3}H-(+)-PN 200-110 to intact cardiac cells. Coverslip cultures of rat cardiac myocytes were equilibrated with increasing concentrations of ^{3}H-(+)-PN 200-110 for 90 min in NaCl- (▲) or KCl-Hanks' medium (●). Unspecific binding in the presence of 1 μM nonlabeled (+)-PN 200-110 has been subtracted. Experimental points were fitted by computerized nonlinear least squares regression assuming binding to a single class of sites and a Hill coefficient of 1. The following K_D (nM) and B_{max} (fmol/mg of protein) values were calculated, respectively, for binding curves in NaCl or KCl medium: 0.73 ± 0.13, 0.059 ± 0.005 (K_D) and 158.9 ± 20.1, 158.3 ± 6.6 (B_{max}). The data give mean values ± standard error of 4–12 cultures from three independent experiments. *Inset:* Total and unspecific binding in K-depolarized cells showing the quantitative relation between specific and unspecific binding.

have a higher affinity for the dihydropyridine channel blockers than channels in the resting or open states.

Competitive displacement of ^{3}H-(+)-PN 200-110 by the (−)-(*R*)- and (+)-(*S*)-enantiomers of 202-791. Previous studies have shown that Ca channel blockers and activators of the dihydropyridine group compete for a common binding site in cardiac cells and cell membrane preparations (23–25). Therefore, it has been concluded that both activating and blocking action of these compounds seem to be mediated by a single dihydropyridine-binding site within the Ca channel (23, 26). Other authors have favored the idea of two functionally distinct receptors within the Ca channel mediating activating and blocking activities of dihydropyridine compounds, respectively (27, 28). Recently, a new 1,4-dihydropyridine derivative (202-791, Sandoz) became available which is closely related to PN 200-110. The two enantiomers of this new compound

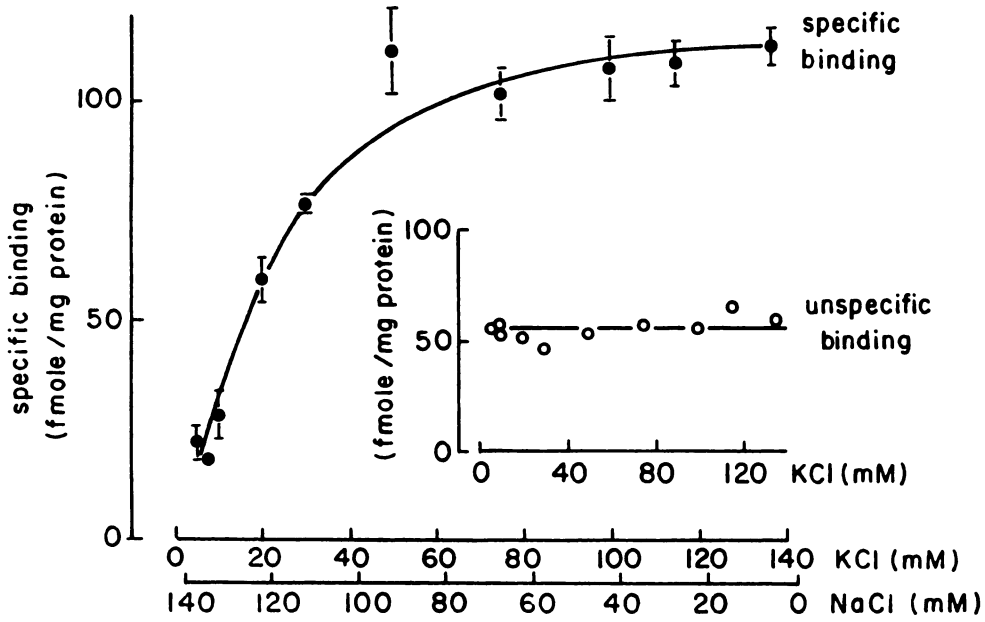

Fig. 9. Effect of stepwise replacement of NaCl by KCl in Hanks' solution on the specific binding of ^{3}H-(+)-PN 200-110 to intact cardiac cells. Coverslip cultures were equilibrated for 90 min with a constant concentration of 0.25 nM ^{3}H-(+)-PN 200-110 in Hanks' solution containing the indicated NaCl and KCl concentrations. Unspecific binding has been subtracted. Experimental points give mean values of four cultures from one experiment. The *curve* was fitted by eye. Two other experiments gave similar results. *Inset*: Unspecific binding as a function of $[K]_o$. Note that unspecific binding was not affected by changing the membrane potential.

showed opposite effects on ^{45}Ca fluxes and Ca-dependent contractions in smooth muscle preparations (7) and on Ca currents and contractile responses in mammalian heart (8). We have used the (*S*)- and (*R*)-enantiomers of 202-791 to assess the "one site hypothesis" for dihydropyridine Ca channel interactions. This hypothesis would predict identical voltage dependence of binding for both activating and blocking isomers. Therefore, in a first set of experiments we have studied competitive displacement of ^{3}H-(+)-PN 200-110 (0.6 nM) by increasing concentrations of (*S*)- and (*R*)-202-791 in polarized and depolarized cardiac cells (Figs. 10–12). In cells with a resting potential of about −40 mV (Fig. 10) the reduction in ^{3}H-(+)-PN 200-110 binding in the presence of the blocking (*R*)-enantiomer was consistent with a 1:1 competition for the same binding site. The displacement curve had a Hill coefficient of 0.9 and the I_{50} value was 2.2 ± 0.6 × 10^{-7} M, corresponding to a K_D value of 1.2 × 10^{-7} M. Two important differences were observed in parallel experiments with the Ca channel-activating (*S*)-enantiomer. First, concentrations of the activator between 3 × 10^{-8} and 10^{-6} M, i.e., below the threshold concentration required for competitive displacement of the radioligand, tended to increase ^{3}H-(+)-PN 200-110 binding. The rather sharp maximum of this increase in specific binding in individual experiments (see Fig. 10, *inset*) is blurred when all experiments of this type are averaged (Fig. 10). An increase was observed in 23 of 26 individual displacement curves from six independent experiments. Radioligand binding rose from 203 ± 5.6 in the absence to 227.9 ± 6.4 fmol/mg of protein in the presence of a maximally effective concentration of (*S*)-202-791 ($n = 26$). The difference was statistically highly significant ($p < 0.001$). Second, with a Hill coefficient of 1.9, the displacement curve obtained with the (*R*)-enantiomer was significantly steeper

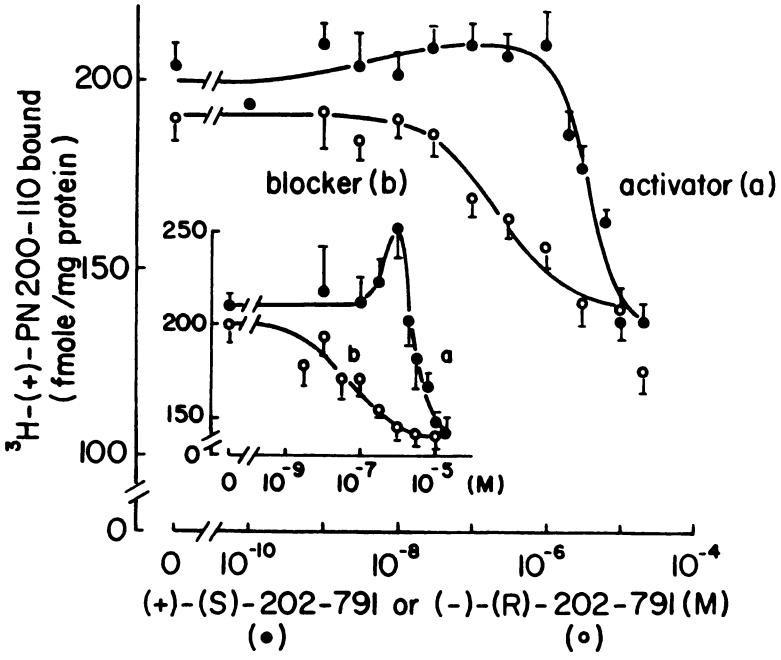

Fig. 10. Competitive displacement of ^{3}H-(+)-PN 200-110 by the Ca channel-activating (+)-(*S*)- and -blocking (−)-(*R*)-enantiomers of the dihydropyridine 202-791 in *polarized* cells. Coverslip cultures were equilibrated for 60 min with 0.6 nM ^{3}H-(+)-PN 200-110 in NaCl-Hanks' containing increasing concentrations of the two enantiomers. The displacement curve for the blocker was calculated by nonlinear least squares regression and yielded an I_{50} value of 0.22 μM corresponding to a K_D value of 0.11 μM. The displacement curve for the activator was fitted by eye. Data points give mean values of 11–26 cultures from six independent experiments. *Inset*: Displacement curves for the blocker (*b*) and the activator (*a*) in a single experiment. The experimental points give the mean of five cultures each.

than that of the activating (*S*)-enantiomer. These two observations suggest that in polarized cells the Ca channel-activating, but not the blocking, enantiomer exhibits positive binding cooperativity with the radiolabeled blocker ^{3}H-(+)-PN 200-110.

Further experiments, summarized in Fig. 11, showed that this allosteric effect was absent in depolarized cells. Cells were equilibrated with 0.075 nM ^{3}H-(+)-PN 200-110, a concentration that corresponds to the K_D value under this condition (Fig. 8). The radioligand was again displaced by increasing concentrations of the two oppositely acting enantiomers of 202-791. The (*S*)-enantiomer was 58-fold less potent than the (*R*)-enantiomer in its ability to compete for ^{3}H-(+)-PN 200-110-binding sites. The Hill coefficients for the two curves were very similar (0.92 and 0.89, respectively). However, compared to the data in Fig. 10, the two curves are shifted significantly to the left, indicating a depolarization-induced increase in the affinity. In Fig. 12 the two sets of data were normalized to facilitate a quantitative comparison of the voltage-induced shifts of the displacement curves. The apparent K_D value for the blocking enantiomer decreased by a factor of 38 from 0.105 μM to 2.25 nM. The shift for the activating enantiomer was smaller. The I_{50} value decreased by a factor of 12. Normalization of the displacement curve for the activator in depolarized cells required that all data points below 1 μM (*S*)-202-791 were averaged and the mean value was set as 100%. Therefore, the increase in ^{3}H-(+)-PN 200-110 binding (compare Fig. 10) in the presence of low activator concentrations is not apparent. Using the Cheng and Prusoff (14) approximation, a K_D value for the (*S*)-enantiomer could be calculated only for depolarized cells. In polarized cells, the affinity of the displaced radioligand increased as a function of the activator concentration (see below). In such a case the Cheng and Prusoff (14) method is not applicable and will result in an erroneously high K_D value for the activator. Consequently, no precise estimate for the voltage-dependent shift in the affinity of the activator can be given. However, the results in Fig. 12 indicate that the K_D for the activator is less voltage sensitive than the one for the blocker.

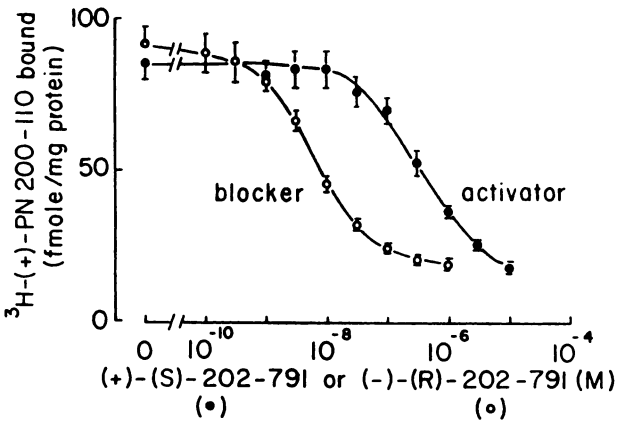

Fig. 11. Competitive displacement of ^{3}H-(+)-PN 200-110 by the (+)-(*S*)- and (−)-(*R*)-enantiomers of the compound 202-791 in *depolarized* cells. Coverslip cultures were equilibrated for 90 min with 0.075 nM ^{3}H-(+)-PN 200-110 and the two enantiomers as described in Fig. 9. The I_{50} values for (−)-(*R*)- and (+)-(*S*)-202-791 as calculated from the regression curve were 5.5 and 320 nM, respectively, corresponding to apparent K_D values of 2.7 and 160 nM. Experimental points give means of 7–11 cultures from three independent experiments.

Stimulation of ^{3}H-(+)-PN 200-110 binding by (+)-(*S*)-202-791. The results shown in Fig. 10 suggest that the (*S*)-enantiomer of the compound 202-791 is an allosteric modulator of ^{3}H-(+)-PN 200-110 binding in polarized cells. However, from these experiments it could not be decided whether the affinity or the capacity of ^{3}H-(+)-PN 200-110 binding sites was affected. If mainly the affinity was altered, one would expect the relative magnitude of the activator-induced increase in ^{3}H-(+)-PN 200-110 binding to become most prominent at low receptor occupancy. Therefore, we have first established a concentration-response curve for (*S*)-202-791 in the presence of a ^{3}H-(+)-PN 200-110 concentration of 0.1 nM corresponding to 0.11–0.17 times its K_D value (0.6–0.9 nM) under these conditions. In the absence of (*S*)-202-791, polarized cells bound 24.3 ± 2.2 fmol of radioligand per mg of protein. Specific binding of ^{3}H-(+)-PN 200-110 reached a peak of 46.8 ± 4.9 fmol/mg of protein with 0.1 μM (*S*)-202-791 (Fig. 13). Analogous experiments with K-depolarized cells in the presence of 0.01 nM ^{3}H-(+)-PN 200-110, i.e., at comparable receptor occupancy, confirmed that the activator had no stimulating effect on ^{3}H-(+)-PN 200-110 binding under this condition (Fig. 13).

The maximally effective concentration of (*S*)-202-791 (0.1 μM) was then used to determine in polarized cells the largest change in ^{3}H-(+)-PN 200-110 binding properties that could be achieved with the activating isomer (Fig. 14). In this group of experiments the mean K_D value for ^{3}H-(+)-PN 200-110 dropped significantly from 0.89 ± 0.18 to 0.2 ± 0.05 nM, whereas the binding capacity decreased from 187.5 ± 27.1 to 129.3 ± 11.4 fmol/mg of protein. In view of the large scatter of the B_{max}

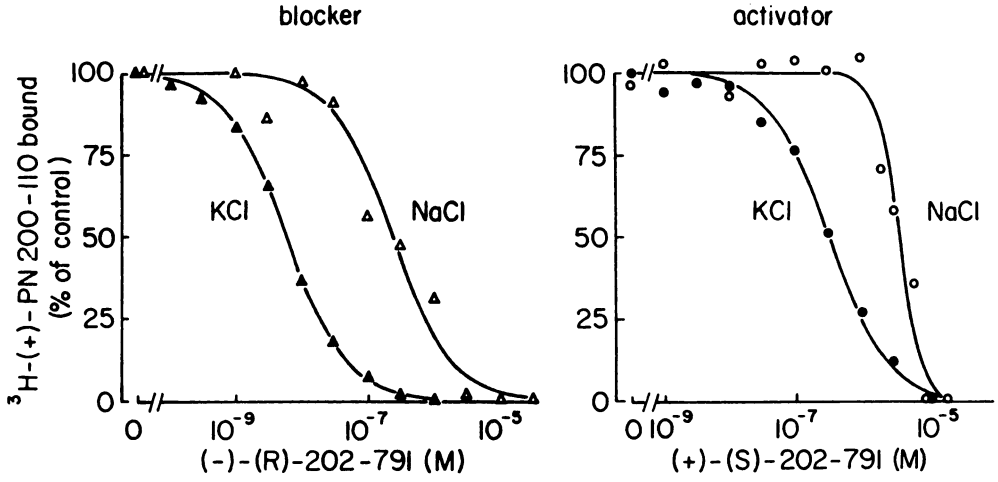

Fig. 12. Normalization of the displacement curves for (+)-(*S*)- and (−)-(*R*)-202-791 from Figs. 9 and 10. NaCl (△, ○) and KCl (▲, ●) denote polarizing and depolarizing conditions, respectively. Except for the displacement of ^{3}H-(+)-PN 200-110 by the activator (○) in NaCl-Hanks' medium (which was fitted by eye), all curves represent nonlinear regressions to the data points assuming Hill coefficients of 1. Depolarization shifted the displacement curves for blocker and activator to the left by factors of 38 and 12, respectively.

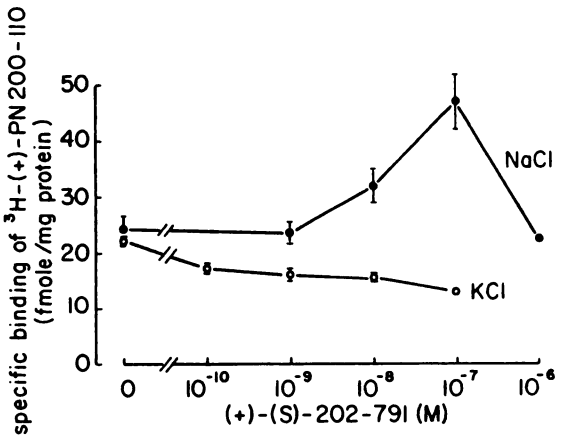

Fig. 13. Stimulating effect of the activator (+)-(*S*)-202-791 on the specific binding of ^{3}H-(+)-PN 200-110 under polarizing (NaCl) but not under depolarizing (KCl) conditions. Coverslip cultures were equilibrated for 60 min with 0.1 nM (NaCl) or 0.01 nM (KCl) radioligand and increasing concentrations of (+)-(*S*)-202-791. Data points give mean values of seven cultures from one experiment. A second experiment gave similar results. Unspecific binding has been subtracted throughout. Note that the initial receptor occupancy in polarized and depolarized cells was not significantly different.

estimates in different groups of experiments under these conditions, the significance of the latter observation remains questionable. Under the same conditions, a 10 nM concentration of the blocking enantiomer increased the K_D value to 1.4 ± 0.7 nM, whereas B_{max} remained unchanged (170 ± 63.6 fmol/mg of protein), as expected for a competitive inhibitor. Such an increase in the binding affinity of the radioligand in the presence of (*S*)-202-791 could, in principle, result not only from allosteric interactions of the two compounds but also from a depolarizing effect of the channel activator. Two observations argue against such a depolarizing effect. First, in control experiments we measured the membrane potential of myocardial cells in ventricle stripes of newborn rat hearts during prolonged exposure to nominally Ca-free Hanks' medium. Under these conditions the resting potential varied between −40 and −50 mV. Joint addition of (+)-PN 200-110 (0.6 or 0.1 nM) and (*S*)-202-791 (1 or 0.1 μM) to the medium had no significant effect on the resting potential during observation periods of up to 1 hr. Second, Ca (1.25 mM) completely suppressed the stimulatory effect of the activator on the binding of ^{3}H-(+)-PN 200-110 even though Ca ions should enhance any depolarizing effect of a channel activator.

In conclusion, these results suggest that the Ca channel activator (*S*)-202-791 promotes binding of the blocker ^{3}H-(+)-PN 200-110 in polarized cells by allosterically increasing its affinity for binding sites associated with the channel.

Effects of Ca^{2+} on the allosteric interactions between Ca channel-blocking and -activating compounds. All experiments described up to this point had been performed in nominally Ca-free incubation media. The mean level of contaminating Ca was determined by atomic absorption spectrophotometry and reached 3–5 μM. Earlier studies with fragmented membrane preparations have shown a stimulating effect of Ca on the binding of 1,4-dihydropyridine derivatives (24, 29, 30). In contrast, functional studies in intact preparations suggest that Ca in mM concentrations may antagonize dihydropyridine effects on Ca channels (20). Therefore, it was of interest to assess a possible Ca effect on the allosteric interactions between (*S*)- and (*R*)-202-791. In a first set of experiments we measured the effect of Ca on the binding of ^{3}H-(+)-PN 200-110 (Fig. 15, *inset*). Increasing the Ca concentrations in the medium from 3 μM to1.25 mM by itself did not change significantly the amount of ^{3}H-(+)-PN 200-110 bound at free ligand concentrations between 0.1 and 1.5 nM. However, Ca changed the effect of (*S*)-202-791 on the binding of the radioligand (Fig. 15). In the nominal absence of Ca, the (*S*)-enantiomer caused the usual increase in binding affinity of the radioligand (compare Fig. 14), whereas the form of the binding curve did not deviate from a single site adsorption isotherm. With 1.25 mM Ca in the extracellular medium, the curve became distinctly sigmoid. Ca reduced ^{3}H-(+)-PN 200-110 binding at free ligand concentrations between 0.05 and 0.5 nM, but did not affect specific binding at concentrations between 0.75 and 1.5 nM. The Hill plot of this binding curve was nonlinear with a maximal slope of $n = 2.47$. If the channel activator had depolarized the cells

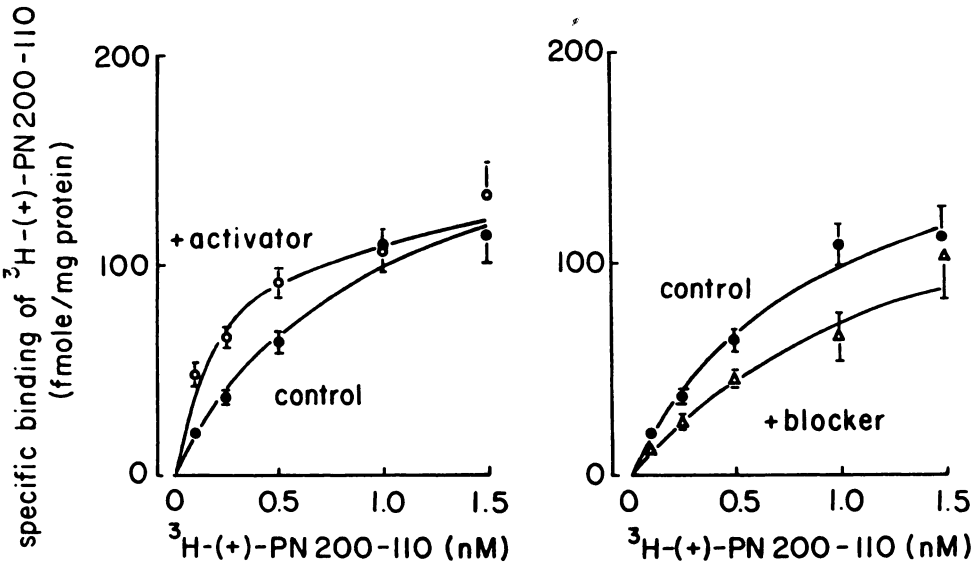

Fig. 14. The activating but not the blocking enantiomer of 202-791 increases the binding affinity of ^{3}H-(+)-PN 200-110. Specific binding of the radioligand was measured under equilibrium conditions in the presence of 0.1 μM (+)-(*S*)-202-791 (*left*) or 0.01 μM (−)-(*R*)-202-791 (*right*). K_D and B_{max} values were calculated as in Fig. 8. The following binding constants were obtained for the binding of ^{3}H-(+)-PN 200-110 under control conditions (●), in the presence of the activator (○), and in the presence of the blocker (△), respectively: K_D (nM), 0.9 ± 0.18, 0.2 ± 0.05, and 1.4 ± 0.68; B_{max} (fmol/mg of protein), 187.5 ±27.1, 129.3 ± 11.4, and 170.0 ± 63.6. Unspecific binding was not affected by the two enantiomers and was subtracted throughout. Experimental points give mean values of 16 cultures from four independent experiments (control), 12 cultures from three independent experiments (activator), and 8 cultures from two independent experiments (blocker).

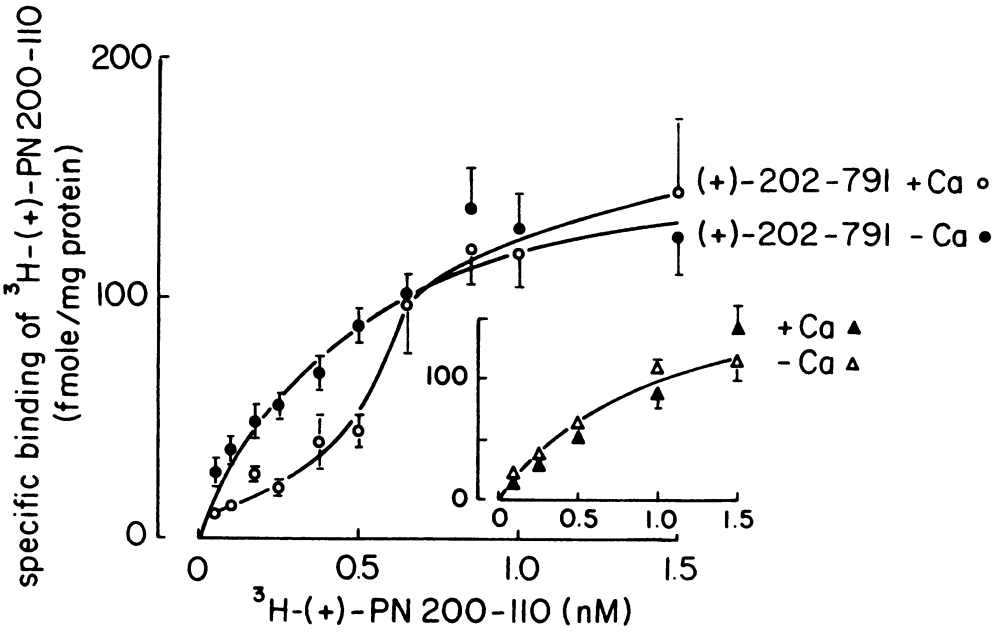

Fig. 15. Effect of Ca on the ^{3}H-(+)-PN 200-110 binding curve in the presence of the activator (+)-(*S*)-202-791. Coverslip cultures were equilibrated for 60 min in NaCl-Hanks' containing 0.1 μM (+)-(*S*)-202-791 together with increasing concentrations of the radiolabel and either 1.25 mM or no added $CaCl_2$. Contaminating Ca in "Ca-free" Hanks' reached about 3 μM. The binding curve in the absence of Ca was calculated as in Fig. 8, K_D = 0.48 ± 0.11, B_{max} = 175.1 ± 21.0. The sigmoid binding curve in the presence of Ca was fitted by eye. Data points give means of 6–10 cultures from three independent experiments. *Inset*: Binding ^{3}H-(+)-PN 200-110 in the presence and absence of 1.25 mM $CaCl_2$. The curve represents the calculated nonlinear regression line fitting the data points for ligand binding in the absence of Ca. Experimental points give mean values of 20–25 cultures from five (−Ca) and six (+Ca) independent experiments.

in the presence of Ca, merely an *increase* in radioligand binding could have resulted, reflecting the decrease in K_D. However, ^{3}H-(+)-PN 200-110 binding at low free ligand concentrations is, in fact, *reduced* and transformed into a sigmoid curve. Hence, these findings suggest, rather, that in the joint presence of Ca and the channel activator (*S*)-202-791, the binding of ^{3}H-(+)-PN 200-110 to polarized cells becomes cooperative. The Ca dependence of this homotropic cooperative effect was quantified in the experiments shown in Fig. 16. Equilibrium binding of ^{3}H-(+)-PN 200-110 at a constant concentration of 0.1 nM was determined as a function of the free Ca concentration in the medium. The Ca-induced difference in the ^{3}H-(+)-PN 200-110 binding curves was maximal at the chosen radioligand concentration (compare Fig. 15). The *left graph* shows the result for polarized cells. Specific binding reached a maximal value of 35.6 ± 7.1 fmol/mg of protein with free Ca concentrations ranging between 3 and 5 μM when the sigmoidicity of the binding curves was minimal or absent. With increasing Ca concentrations binding was progressively reduced until a plateau value of ~14 fmol/mg of protein was reached with 500 μM free extracellular Ca. Lowering the Ca concentration to 0.3 and 0.01 μM by using EGTA as a Ca buffer also reduced specific binding of ^{3}H-(+)-PN 200-110. However, under these conditions a direct effect of EGTA on the binding of ^{3}H-(+)-PN 200-110, in addition to the effect of lowering the free Ca concentration, could not be definitely excluded. By contrast, in depolarized cells (Fig. 16, *right*), at about the same fractional receptor occupancy, Ca was without effect on ^{3}H-(+)-PN 200-110 binding. Hence, allosteric modification of the dihydropyridine-binding sites by Ca in the presence of the channel activator must itself be voltage dependent.

Discussion

In our study we have compared the modulation of electrophysiological properties of Ca channels by dihydropyridines

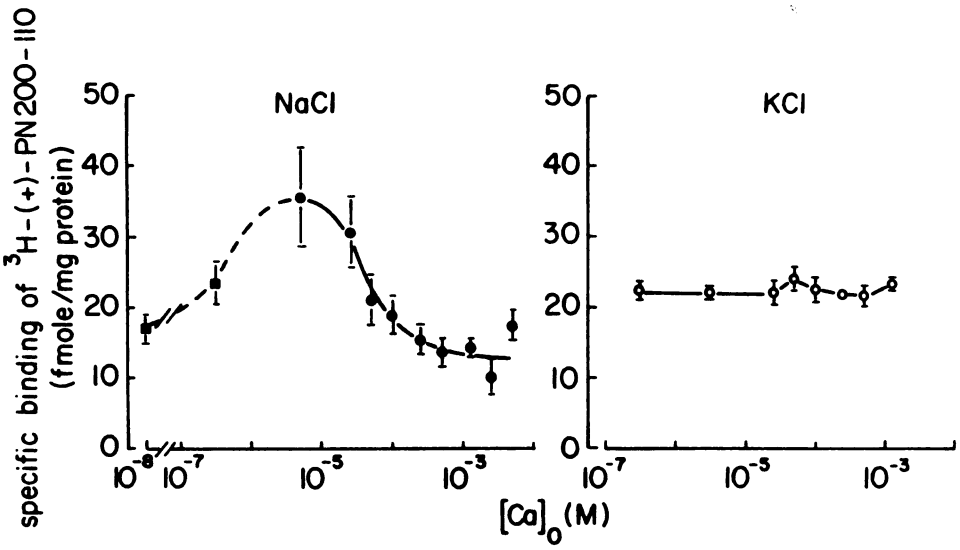

Fig. 16. Effect of Ca on the specific binding of ^{3}H-(+)-PN 200-110 in the presence of the activator (+)-(*S*)-202-791 (0.1 μM) in polarized (NaCl) and depolarized (KCl) cells. Same experimental conditions as in Fig. 14. Concentration of the radioligand was 0.1 nM for polarized cells and 0.01 nM for depolarized cells. Data points for polarized cells (●, ■) give the mean of nine cultures from two independent experiments; those for depolarized cells (○) give the mean of six cultures from one experiment. *Left*: ●—● (polarized cells, line fitted by eye), experimental medium contained no Ca-buffer. ■– – –■, experimental medium contained EGTA-buffered Ca concentrations (EGTA concentration 0.1 mM). Two additional but less complete experiments with polarized cells and one additional experiment with depolarized cells gave similar results. Note that the decrease of specific binding with Ca concentrations increasing from 3 μM to 5 mM is a direct consequence of the increasing sigmoidicity of the ^{3}H-(+)-PN 200-110 binding curve under these conditions (compare Fig. 15).

with direct binding of the drugs to these channels. We could demonstrate that the binding affinity, but not the binding capacity, of dihydropyridines increases when intact cardiac cells are depolarized. Earlier electrophysiological evidence has shown that partial depolarization of the membrane potential enhances the potency of dihydropyridines to block cardiac Ca channels by up to 3 orders of magnitude (4–6). These findings were explained by assuming that blockers bind with high affinity to inactivated Ca channels but only with low affinity to channels in the normal resting state. This explanation would resolve at least some of the discrepancy between the low K_D values for dihydropyridines in cardiac membrane homogenates and the 100–1000-fold higher concentrations required for macroscopic pharmacological effects in the intact tissue (20, 31, 32).

Attempts by others to measure directly the effect of depolarization on dihydropyridine binding in intact cardiac myocytes have failed to detect a change in binding affinity. Instead, a significant increase in binding capacity was observed (33). By contrast, our binding studies confirm the predictions made on the basis of electrophysiological experiments. What is the reason for these conflicting results? It is important to note that we have compared ligand binding to confluent myocardial cell layers with homogeneous membrane potentials of about −38 and 0 mV, respectively. At the rather low membrane potential of −38 mV the apparent binding affinity is presumably much higher than at membrane potentials around −80 mV (4). At 0 mV the K_D value (0.06 nM) characterizes binding to a homogeneous population of inactivated channels and agrees with the corresponding value (0.064 nM) in rat cardiac homogenates (34). The maximal binding capacity for ^{3}H-(+)-PN 200-110 in intact cells (150–200 fmol/mg of total cell protein) also agrees with the value reported for crude homogenates (148 fmol/mg of protein) (34).

With the technique used in the present experiments it would be impossible to measure reliable binding constants of ^{3}H-(+)-PN 200-110 (or of any other known ^{3}H-labeled dihydropyridine) to cells with normal resting potentials: specific binding of dihydropyridines to intact cells can be measured only when ligand concentrations do not exceed 2 nM. High unspecific binding prevents the use of higher concentrations. Green *et al.* (33) used dissociated cardiac cells which presumably had very variable membrane potentials between −80 mV in healthy cells and 0 mV in damaged cells. They observed an increase in B_{max} that can be explained by an increase in binding affinity. Damaged or dead cells without membrane potential will bind the radioligand with high affinity, whereas in intact cells specific and unspecific binding cannot be separated. However, depolarization of intact cells with KCl would convert low affinity binding sites into high affinity sites with K_D values similar to that in damaged cells. This will appear as an apparent increase in B_{max}.

Interactions between Ca channel-activating and -blocking enantiomers of compound 202-791. The discovery of 1,4-dihydropyridines with Ca channel-activating effect has led to a debate whether more than one specific high affinity binding site for dihydropyridines exists in functional Ca channels (6, 27, 28). In one model dihydropyridines are viewed as agonists, partial agonists, or antagonists interacting at a single site with different "intrinsic activities" in analogy to agonist/antagonist actions at hormone receptors (35, 36). Up to now, binding experiments have not provided direct evidence for a second "activating" dihydropyridine-binding site. Most studies report competitive interactions between activators and blockers of the dihydropyridine type suggesting 1:1 competition for the same high affinity binding site at Ca channels (23, 24). In addition, low affinity binding sites for activators and blockers have been observed, but their association with activating or blocking activities of dihydropyridines remains unclear (24, 37). The situation is further complicated by the fact that ^{3}H-Bay K 8644, the only dihydropyridine Ca channel activator available for binding studies, is a racemate of two oppositely acting enantiomers (38). (+)- and (−)-Bay K 8644 differ only marginally in their apparent affinities for the dihydropyridine receptor (39).[1]

The positive inotropic and Ca current-stimulating actions of channel activators are fairly well correlated with their apparent K_D values in membrane homogenates and intact cells (16, 23, 40, 41). These observations indicate that the affinity of activators to channels in the resting or open state is high and that inactivation affects the affinity of activators less than that of blockers. This is in agreement with the smaller effect of depolarization on the I_{50} values for the activator in competitive displacement experiments (Fig. 12).

Our results demonstrate positive cooperativity between the pure enantiomers (+)-(*S*)-202-791 (activator) and ^{3}H-(+)-(*S*)-PN 200-110 (blocker) and, therefore, suggest that in polarized cells more than one high affinity binding site can be occupied simultaneously by dihydropyridine Ca channel ligands. Binding and allosteric coupling between binding sites are both voltage dependent. Hence, the binding reaction probably takes place at structural components of the channel which are involved in the potential-dependent gating process.

There are two problems with the interpretation of our data. 1) We could only study the effects of a channel activator on the binding of a radiolabeled blocker, but we know very little of possible effects of the blocker on binding of the activator. Therefore, properties of the activator-binding site can only be indirectly inferred (compare Table 1). However, these results of our functional studies suggest that the mutual interaction of blocking and activating dihydropyridines is at least partially symmetric. The activator-induced increase in the binding affinity of the blocker seems to be paralleled by a blocker-induced increase in the affinity of the activator resulting in their synergistic stimulation of Ba currents (Fig. 7). 2) All of our binding experiments have been performed under equilibrium conditions

TABLE 1

Properties of two Ca channel-binding sites for channel activators and blockers of the dihydropyridine group

	Polarized cells	Depolarized cells
Affinity for blocker		
at activating site	low	low
at blocking site	low[a]	high[a]
Affinity for activator		
at activating site	high	low
at blocking site	low[a]	high[a]
Cooperativity between activating and blocking sites	strong	not measurable
Cooperativity between Ca site, activating site, and blocking site	strong	not measurable

[a] Equilibrium binding constants measured in this study.

[1] H. Porzig and C. Becker, unpublished results.

and provide no information on binding kinetics. Equilibrium binding data are not sufficient to characterize dihydropyridine binding in terms of one of the specific models for allosteric interactions which take transient state kinetics into account (42). Nevertheless, several interesting analogies can be found between our observations and the allosteric systems analyzed by Monod *et al.* (43), or Koshland *et al.* (44). These models were developed originally for a quantitative treatment of steady state enzyme kinetics in the presence of allosteric effectors. The same formal treatment is applicable to receptor binding studies under equilibrium conditions (42, 45). Allosteric enzyme activators or inhibitors and the enzyme substrate have their analogies in channel activators, blockers, and ions.

The model of Monod *et al.* (43) (MWC model) considers a protein that has at least two symmetrical stereospecific ligand-binding sites and can exist in at least two interconvertible conformational states. The state transition is associated with a change of the affinity of the binding sites toward the corresponding ligand. Cooperative effects are assumed to be exclusively due to the ligand-induced displacement of the spontaneous equilibrium between the different conformations of the protein.

Under our conditions, the spontaneous equilibrium between the possible conformational states of the Ca channel and the associated dihydropyridine-binding sites depends primarily on the steady membrane potential rather than on ligand binding. The potential determines the probability that the channel protein will assume one of three defined states, resting (R), open (O), or inactivated (I), with different affinities for activators and blockers. Electrophysiological evidence suggests that the channel can exist in multiple closed states which are lumped together in state R and which are beyond the resolution of binding studies. A model system in which the state change of an ion channel leads to conformational changes of a drug receptor has been analyzed by Hille (46) and by Hondeghem and Katzung (47). This "modulated receptor" hypothesis has been used to describe voltage-dependent interactions of local anesthetics with Na channels and of dihydropyridines with Ca channels (4, 5, 48). However, this concept does not account for cooperative interactions between different ligands, although the "modulated receptor" can be considered as a special case of an allosteric protein. Even closer analogies exist between allosteric effects of dihydropyridines at cardiac Ca channels and the heterotropic cooperative interactions of alkaloid neurotoxins with scorpion toxin at the Na channels of neuroblastoma cells described by Catterall (49).

Allosteric effects could arise in a voltage-modulated receptor system if the binding of dihydropyridines to one of the two high affinity sites induces small voltage-independent shifts in the steady state frequency distribution for the different channel conformations. Such an effect is likely to be maximal under conditions where the probability for spontaneous state transitions is high. This is the case in partially depolarized cells. At a steady potential of −38 mV as in our experiments, the majority of Ca channels will exist in the R-state. Yet, the probability of R→I transitions will be significantly higher than at more negative potentials. Similarly the probability of I→R transitions will be higher than at more positive potentials. In contrast, allosteric effects due to ligand binding will be minimal or absent when the probability of spontaneous state transitions is low, as in depolarized cells. The available experimental evidence is consistent with this hypothesis.

Functional studies in cardiac and smooth muscle cells indicate that channel activators may stimulate Ca fluxes at membrane potentials near the threshold for Ca channel activation (40, 50, 51). This effect would increase the number of channels cycling through O- and I-states and, hence, increase the population of high affinity binding sites for blockers without apparent change in membrane voltage.

The results of our own electrophysiological experiments confirm this prediction. They show that the activator shifts significantly the steady state inactivation curve to more negative potentials and enhances the rate of voltage-dependent Ca current inactivation (Fig. 4).

Conversely, it has frequently been observed that small concentrations of blockers can increase the open state probability of the channel (6, 16, 17). This was also true for the (*R*)-enantiomer of 202-791. The effect has been interpreted as "partial agonistic" activity of these compounds (36). Other reports have shown "paradoxical" potentiation of smooth muscle contractile responses to the activator BAY K 8644 by the blocking compounds nitrendipine and nimodipine (28, 52). The present study demonstrates a potentiating effect of the blocking enantiomer (*R*)-202-791 on the (*S*)-202-791-induced increase in open states of the channel. Taken together, this evidence suggests that, under certain conditions, channel blockers may increase rather than decrease channel open states and, consequently, promote high affinity binding for an activator.

The MWC-model requires two identical binding sites on a dimeric protein as the minimal condition for allosteric interactions. Would a similar assumption be consistent with our experimental findings? If two identical allosterically coupled binding sites for dihydropyridines exist which merely differ in their affinities for activating and blocking compounds, the following conditions must be met: 1) activator and blocker should compete for the same binding sites both in polarized and depolarized cells; 2) homotropic and heterotropic allosteric effects should be observed for activators and blockers; and 3) binding site density should be similar for blockers and activators and should be voltage independent.

Since it was not possible to test reciprocal interactions between activators and blockers of Ca channels, we do not know to what extent symmetry requirements are met. The observation of homotropic cooperative effects of the blocker in the joint presence of Ca and the activator is a strong argument in favor of two closely related sites both being simultaneously accessible to the blocker. Conversely, the different potential sensitivity of blocker and activator binding affinities and the lack of homotropic cooperativity in the absence of Ca are not compatible with the assumption of a complete symmetry of sites. In depolarized cells, we observed a single K_D value for the radiolabeled blocker and 1:1 competition between blocker and activator. This is possible if the two sites look identical for both blockers and activators, or if the affinity of the activator site for the blocker is too low to be detected in our binding assay. Identical binding capacities for ^{3}H-(+)-PN 200-110 in polarized and depolarized cells argue in favor of the second possibility.

In Table 1 we have summarized some qualitative differences between the two Ca channel binding sites that can be inferred

from our studies on the voltage dependence of competitive interactions between activating and blocking dihydropyridines.

In addition to membrane potential changes, $[Ca]_0$ seems to be capable of modulating the conformation of the dihydropyridine-binding site. The Ca-induced homotropic cooperative binding of the radiolabeled blocker may be a consequence of the more general effects of this ion on Ca channel properties. Electrophysiological studies in cardiac muscle (53) and skeletal muscle (54) have shown that Ca is involved in the regulation of ion selectivity of voltage-dependent Ca channels. In the absence of $[Ca]_0$ the channel becomes permeable for monovalent cations. Increasing $[Ca]_0$ confers divalent cation selectivity (apparent K_D for Ca^{2+}, 0.7–2 μM), and, hence, must be able to induce some change in channel conformation. In our experiments the sigmoidicity of the ^{3}H-(+)-PN 200-110 binding curve in the presence of (*S*)-202-791 was lost near the K_D of the high affinity Ca-binding site. Hence, homotropic cooperativity was probably minimal under this condition but increased when this site was either empty or fully occupied.

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